

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



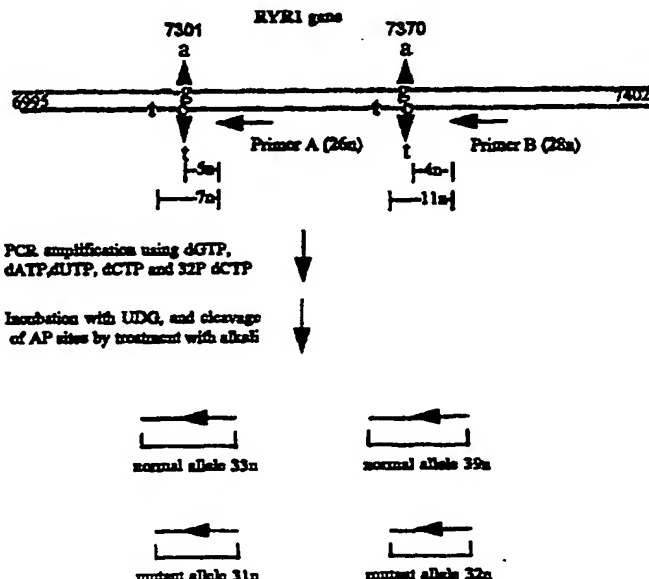
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68	A1	(11) International Publication Number: WO 97/03210
		(43) International Publication Date: 30 January 1997 (30.01.97)
<p>(21) International Application Number: PCT/IE95/00067</p> <p>(22) International Filing Date: 21 December 1995 (21.12.95)</p> <p>(30) Priority Data: 950526 11 July 1995 (11.07.95) IE</p> <p>(71) Applicants (for all designated States except US): FORFÁS (trading as BIORESEARCH IRELAND) [IE/IE]; Wilton Park House, Wilton Place, Dublin 2 (IE). UNIVERSITY COLLEGE CORK [IE/IE]; College Road, Cork (IE).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): McCARTHY, Thomas, Valentine [IE/IE]; Vista Villa, Montenotte, Cork (IE). VAUGHAN, Patrick, Martin [IE/IE]; 15 Westbury Grove, Wilton, Cork (IE).</p> <p>(74) Agent: ANNE RYAN & CO.; 60 Northumberland Road, Ballsbridge, Dublin 4 (IE).</p>		<p>(81) Designated States: AU, CA, JP, NO, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published With international search report.</p>

(54) Title: GLYCOSYLASE MEDIATED DETECTION OF NUCLEOTIDE SEQUENCES AT CANDIDATE LOCI

(57) Abstract

A method for rapidly detecting the presence or absence of a particular nucleic acid sequence at a candidate locus in a target nucleic acid sample comprises the steps of: i) introducing a modified base which is a substrate for a DNA glycosylase into said candidate locus at one or more preselected positions; ii) excising the modified base by means of said DNA glycosylase so as to generate an abasic site; iii) cleaving phosphate linkages at abasic sites generated in step ii); and iv) analysing the cleavage products of step iii) so as to identify in said target nucleic acid sequence the presence or absence of said particular nucleic acid sequence at said candidate locus. The method has particular application for detecting specific mutations in a DNA sample, including the detection of multiple known mutations in DNA. Large throughput of sample can be achieved rapidly and easily.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

Glycosylase mediated detection of nucleotide sequences at
candidate loci

Technical Field

5 This invention relates to a method for rapidly detecting the presence or absence of a particular nucleic acid sequence at a candidate locus in a target nucleic acid sample. In particular, the invention relates to a method for detecting specific mutations in a DNA sample.

Background Art

10 Detection of specific sequences at candidate loci in target nucleic acid samples is highly important for several reasons relating to diagnosis of inherited disorders and of infectious diseases. Detection of multiple different mutations is necessary for screening for the presence of specific genetic disorders. Detection of specific sequences in amplified DNA samples significantly enhances current DNA diagnostic
15 methods for the detection of organisms of interest and especially of infectious organisms. Current techniques for the detection of sequences at candidate loci are either cumbersome, lacking in specificity, difficult to optimise and use or poorly adaptable to high sample throughput.

20 There are several methods known for the detection of a particular nucleic acid sequence at a candidate locus in a target nucleic acid sample. Details of these methods are as follows.

1) Restriction enzyme analysis

25 Detection of a particular DNA sequence by restriction enzyme analysis. Restriction enzymes cleave DNA at specific sequences. For example, the enzyme EcoRI cleaves double stranded DNA at the sequence GAATTC. Thus, by checking a candidate locus in a particular DNA sample for cleavage with EcoRI, one may determine whether the sequence GAATTC is present or absent at the candidate locus. The appearance or disappearance of a restriction site from a

candidate locus indicates that one or more of the bases in the sequence at the candidate locus has been altered. Thus, the creation or loss of a restriction site at a candidate locus can involve the alteration of any base or bases of the respective restriction site. Therefore, the appearance or disappearance of a restriction site at a candidate locus may not inform the investigator of the exact sequence change. However, there are a number of problems with the use of restriction enzymes for the detection of specific sequences at candidate loci. These include:

- a) the presence or absence of a specific sequence at a candidate locus regularly does not occur at a restriction site;
- b) since different restriction enzymes have different recognition sequences, different restriction enzymes are regularly required for the detection of the presence or absence of a particular sequence at different candidate loci; and
- c) because different enzymes can be required for the detection of the presence or absence of a particular sequence at different candidate loci, a high throughput is difficult to achieve with this approach and automation is difficult.

2) DNA sequencing

DNA sequencing allows detection of a particular sequence at any candidate locus. The main methods of DNA sequencing are the Sanger method (Sanger, F. and Coulson, A.R. (1975) J. Mol. Biol. 94, 444-448), also known as the dideoxy method or chain termination method, and the Maxam Gilbert method (Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564), also known as the chemical method.

- A method has been described whereby uracil is incorporated into an amplified DNA molecule randomly and at a low level. This is achieved by amplifying the DNA in the presence of the normal DNA precursor nucleotides and dUTP. The ratio of dTTP to dUTP is

25

chosen so that in the amplification process dUTP is occasionally incorporated opposite an adenine residue on the template strand while dTTP is incorporated opposite adenine residues at a much higher frequency. This results in a population of products bearing a low level of uracil residues randomly distributed throughout the amplified molecules. Treatment of the amplified products with uracil glycosylase and cleavage of the abasic site results in cleavage of the molecules at the position of incorporation of the uracil residues. Because the uracil was incorporated randomly opposite adenine residues at a low level, different molecules will be cleaved at different points depending on where the uracil residues were incorporated. Thus labelling of one of the primers used in the amplification process and separation of all of the cleavage products on a DNA sequencing gel produces a ladder of fragments that allows the determination of the total number of positions of uracil incorporation in one strand of the amplified DNA sample (Tu, W.T. and Struhl, K. Nuc. Acids Res., (1992) 20, 771-775. Devchand, P.R. *et al.* Nuc. Acids Res. (1993) 21, 3437-3443).

The main application of the approach outlined has been for DNA footprinting (a method used to identify the bases in DNA to which particular proteins bind).

The uracil incorporation method can be used to determine the location of the total number of uracil residues in an amplified DNA sample. However,

- a) it would be a cumbersome and difficult method for rapid detection of an uracil residue at a specific candidate locus;
- b) full size sequencing gels would be required for determination of an uracil residue at a specific candidate locus;
- c) sequencing gels can often be difficult to read/interpret; and

- d) resolution of multiple DNA fragments of different size would be necessary to detect an uracil residue at a specific candidate locus.

4) Use of mismatched nucleotide glycosylases

- 5 Two accounts of the use of mismatched nucleotide glycosylases have been reported for detection of point mutations (Lu, A-L and Hsu, I-C, Genomics (1992) 14, 249-255 and Hsu, I-C., *et al*, Carcinogenesis (1994)14, 1657-1662). The glycosylases in question are the *E. coli* Mut Y gene product which releases the mispaired adenines of A/G mismatches efficiently and A/C mismatches inefficiently by regular glycosylase action and the human thymidine DNA glycosylase which cleaves at G/T mismatches. These enzymes have been used for mutation detection in amplified heteroduplex DNA molecules where mismatches are present. Labelling of one of the primers used in the amplification reaction permits detection of the position of the mismatch after glycosylase treatment, cleavage of the abasic site and resolution of the fragments by gel electrophoresis.

There are several problems with this method as follows:

- 20 a) the method is dependent on the formation of heteroduplex molecules where a mismatched based pair is formed at the site in question. Thus, to detect a mutation in a homozygous sample, an external probe must be provided and hybridization carried out to generate the mismatch;
- 25 b) the method permits detection of the position of the mismatch but not necessarily the sequence at the mismatch; and
- c) not all mismatches are recognised with equal efficiency.

5) Other methods based on cleavage of mismatched base pairs

Several other methods based on the cleavage of mismatched base pairs have been described for detecting point, deletion and insertion type mutations. These include chemical cleavage at mismatched base pairs, heteroduplex detection based on the slower migration thereof during gel electrophoresis relative to homoduplex molecules which migrate faster, RNase cleavage of mismatches in RNA:DNA hybrids and cleavage of mismatches by enzymatic means.

All of the above methods can detect mutations in heteroduplex molecules and allow the approximate position of the mutation in the nucleic acid molecule to be determined (except in the case of the heteroduplex electrophoresis retardation method which only informs of the presence of a mutation in the sample). However, these methods only work on heteroduplex DNA and do not allow one to deduce what specific sequence is present at a candidate locus.

6) Ligase chain reaction

The ligase chain reaction (LCR) is a probe amplification method that can be used for detecting the presence or absence of a particular target sequence at a candidate locus. It utilises the enzyme DNA ligase to join two pairs of oligonucleotides that hybridise adjacent to one another on the denatured target DNA strands. The enzyme forms a phosphodiester link between the two oligonucleotides, provided that the oligonucleotides at the junction correctly hybridised with the template. Thus an exact match between the oligonucleotides and the target sequence at the junction permits ligation of the oligonucleotides resulting in the formation of a larger product which is the cumulative size of both the oligonucleotides. Multiple cycles of annealing, ligation and denaturation results in the exponential amplification of the larger product. Thus detection of the larger product indicates the presence of a sequence at the candidate locus while absence of the larger product

means that there were differences between the oligonucleotide sequences and the sequence of the template DNA.

While this method has good potential for detecting particular sequences at candidate loci and also offers high throughput of samples, it does not allow one to deduce what specific sequence is present at a candidate locus. In addition, the method requires considerable effort to optimise the process.

7) ARMS method

By designing appropriate primers for use in the polymerase chain reaction, it is possible to detect the presence or absence of a specific sequence at a candidate locus. In this method known as the amplification refractory method (ARMS), primers are designed so that amplification of a target sequence only occurs if there is a perfect match between the 3' end of the primers and the target sequence. Thus if a pair of primers are designed so that one primer is complementary to a given sequence of the target sample while the partner primer is designed so that its 3' end is complementary to the wild type sequence at the candidate locus, then this pair of primers will only produce a product on amplification if the wild type sequence is present at the candidate locus. If a third partner primer is designed so that its 3' end is complementary to the mutant sequence at the candidate locus, then this primer if used with the primer complementary to the given sequence of the target sample will only produce a product on amplification if a mutant sequence is present at the candidate locus. This method suffers from a number of problems as follows:

- a) three primers are required to determine whether wild type or mutant sequence is present at the candidate locus;
- b) the method does not allow one to deduce what specific sequence is present at a candidate locus; and

- c) the annealing conditions for the ARMS method have to be precise, thus the method is difficult to transfer in many cases and has to be optimised for each mutation investigated.

5 Thus, it will be appreciated that there is a need in the nucleic acid diagnostics field for a robust method for detection of specific sequences at candidate loci that allows rapid and high throughput of samples.

Disclosure of Invention

10 The invention provides a method for rapidly detecting the presence or absence of a particular nucleic acid sequence at a candidate locus in a target nucleic acid sample which comprises the steps of:

- i) introducing a modified base which is a substrate for a DNA glycosylase into said candidate locus at one or more preselected positions;
- 15 ii) excising the modified base by means of said DNA glycosylase so as to generate an abasic site;
- iii) cleaving phosphate linkages at abasic sites generated in step ii); and
- 20 iv) analysing the cleavage products of step iii) so as to identify in said target nucleic acid sequence the presence or absence of said particular nucleic acid sequence at said candidate locus.

25 The method according to the invention offers significant advantages over existing methods in that a single enzyme and a single process can be used to detect multiple known mutations in DNA. Thus, a large throughput of sample can be achieved rapidly and easily as hereinafter demonstrated.

The method in accordance with the invention enables one to investigate a target nucleic acid to determine if a particular sequence, such as a gene mutation is presence or absent at a particular location known as the "candidate locus" herein.

5 Preferably, the candidate locus is amplified using normal DNA precursor nucleotides and at least one modified precursor nucleotide.

 The term "amplifying" as used herein refers to any *in vitro* process for increasing the number of copies of a nucleotide sequence or sequences. Amplification of a target sample results in the
10 incorporation of precursor nucleotides into the DNA being amplified. Typically, amplification of a target sample is carried out using appropriate primers in the polymerase chain reaction (PCR). Amplification of a target sample may be also carried out using the
15 ligase chain reaction (LCR) and a variation of the LCR which employs a short PCR step (PLCR). Precursor nucleotides in the case of a DNA amplification process refer to the deoxyribonucleotides dATP, dCTP, dGTP and dTTP herein referred to as "normal" DNA precursor nucleotides. Modified precursor nucleotide(s) as used herein refers to
20 a modified nucleotide or nucleotides that can be incorporated into a nucleic acid so that a substrate base or bases (glycosylase substrate base) is generated which is recognised by a DNA glycosylase enzyme.

 The amplification will typically involve amplifying a target nucleic acid sample using a combination of normal DNA precursor nucleotides and one or more modified precursor nucleotide(s) where
25 the modified precursor nucleotide replaces one of the normal precursor nucleotides. The incorporation of a modified precursor nucleotide into the amplified product generates one or more glycosylase substrate base(s) at one or more positions recognised by a DNA glycosylase enzyme in the amplified product. A particular sequence may be
30 present at the candidate locus in all or a portion of the target nucleic acid sample, or may be absent from the target nucleic acid sample.

Further, preferably, at least one of the primers for the amplification is positioned adjacent to the candidate locus.

Thus, suitably primers for amplification purposes are designed such that one of the primers is positioned adjacent (referred to as the "adjacent primer" herein) to the candidate locus so that during the amplification process, the position of incorporation of the first modified precursor nucleotide into the extended adjacent primer will be at, or distal to, the candidate locus depending on the particular sequence present at the candidate locus. The other primer of a pair of primers as used herein is referred to as the "distal primer" as hereinafter defined.

Design of the adjacent and distal primers so that the modified precursor nucleotide is incorporated at the candidate locus alone or not incorporated at all, if a particular sequence is present or absent in the amplified target nucleic acid sample, permits cleavage of the target strand bearing the glycosylase substrate base into two fragments.

Amplification of the target strand of the target sample can be achieved using the adjacent oligonucleotide primer which anneals to the complementary region of the complementary target strand of the target nucleic acid sample. Primer extension of the adjacent primer in the amplification process results in the incorporation of precursor nucleotides and modified precursor nucleotides in a 5' to 3' direction. The amplified DNA strand generated through the extension of the adjacent primer in the amplification process is referred to as the "target strand" herein. The amplified DNA strand generated through the extension of the other primer (referred to as the "distal primer" herein) in the amplification process is referred to as the "complementary target strand" herein. Amplification of the complementary target strand of the target sample can be achieved using the distal oligonucleotide primer which anneals to the complementary region of the target strand of the target nucleic acid sample. Primer extension of the distal primer in the amplification process results in the incorporation of precursor nucleotides and modified precursor

nucleotides in a 5' to 3' direction. Amplification of both strands of the target sequence can be achieved using both the adjacent and distal primers. In the PCR process, repeated cycles of amplification are performed. The size of the amplified fragment in this case is
5 delineated by the position of annealing of the adjacent and distal primers to the strands of the target nucleic acid sample.

For detection of a particular sequence such as a mutation at the candidate locus in a target sample, primers for amplification purposes are designed so that the adjacent primer is positioned close to the
10 candidate locus so that during the amplification process, the position of the first modified precursor nucleotide incorporated into the extended adjacent primer will be at, or distal to, the candidate locus depending on whether a particular sequence is present or absent at the candidate locus. In cases where the incorporation of a sole modified precursor
15 nucleotide at the candidate locus is desirable, the primers are designed so that all of the bases in the primers promote preferential incorporation of nucleotides other than the key modified precursor nucleotide (the key modified precursor nucleotide being the modified precursor nucleotide to be incorporated at the candidate locus
20 depending on the presence or absence of a particular sequence) in the newly synthesised DNA complementary to the primer sequences. For example, in the case where dUTP is the modified precursor nucleotide, the primers are synthesised so that the adenine bases are replaced by cytosine, guanine, thymine, inosine or modified bases (other than
25 uracil) which preclude incorporation of uracil residues in the newly synthesised DNA complementary to the primer sequences. In the case where dTTP is the modified precursor nucleotide, the primers are synthesised so that the cytosine bases are replaced by guanine, thymine, adenine, uracil or modified bases (other than inosine) which preclude
30 incorporation of inosine residues in the newly synthesised DNA complementary to the primer sequences. In cases where cleavage at specific points in the primers is desirable in addition to cleavage at the candidate locus on the target strand, the primers are synthesised so that one or more glycosylase substrate base(s) is/are present in the primer at
35 a defined position or positions. Treatment of the primer with the

appropriate glycosylase after the amplification process will result in cleavage of the primers at a specific position or positions. Design of the primers in such a fashion facilitates detection of specific fragments of the target strand indicative of the presence or absence of a particular sequence at the candidate locus and serves to reduce the size of primers if desirable so that they do not interfere with the detection process.

Suitably at least one primer is labelled when an amplification method is used in accordance with the invention. Labelling of one of the primers prior to the amplification process allows detection of the amplified target or complementary strand alone. Labelling of the primers can be performed by a variety of means including addition of a radioactive, fluorescent, or detectable ligand to the primer during or post primer synthesis. The use of a labelled precursor nucleotide (i.e. a radioactive precursor nucleotide, or a precursor nucleotide with a linked fluorescent or detectable ligand group) in the amplification process facilitates detection of the target strand and the complementary target strand and any DNA fragments arising from the glycosylase mediated cleavage process that bear the incorporated label. DNA staining methods such as silver or ethidium bromide staining facilitates detection of all of the fragments generated as a result of the glycosylase mediated cleavage of the amplified target nucleic acid sample after separation of the fragments. Alternatively, detection of the amplified target and complementary strand and fragments generated as a result of the glycosylase mediated cleavage can be accomplished using appropriate nucleic acid hybridisation probes.

The modified base can be introduced by chemical modification of an existing base at the candidate locus.

Several methods exist where treatment of DNA with specific chemicals modify existing bases so that they are recognised by specific DNA glycosylase enzymes. For example, treatment of DNA with alkylating agents such as methylnitrosourea generates several alkylated bases including N3-methyladenine and N3-methylguanine which are recognised and excised by alkyl purine DNA-glycosylase. Treatment

of DNA with sodium bisulfite causes deamination of cytosine residues in DNA to form uracil residues in the DNA which can be cleaved by uracil DNA-glycosylase .

Thus, from knowledge of the prior art, bases present at the candidate locus of an amplified target nucleic sample can be converted into glycosylase recognisable substrates by chemical means. For example, a cytosine present at the candidate locus can be readily converted into an uracil, thereby rendering the amplified sample susceptible to uracil DNA-glycosylase cleavage at the candidate locus. If the adjacent primer is synthesised so that it contains 5-methylcytosine rather than cytosine in such a case, the primer will be resistant to uracil DNA-glycosylase mediated cleavage since deamination of 5-methylcytosine generates a thymine residue rather than an uracil residue.

Preferably, the modified base is excised (or cleaved) by means of a DNA glycosylase enzyme.

Thus, suitably following the amplification process, the amplified product is treated with a suitable DNA glycosylase enzyme which recognises and releases the glycosylase substrate bases present in the amplified target sample and consequently generates apurinic or apyrimidinic sites in the amplified target nucleic acid sample.

In the case where the modified precursor nucleotide is dUTP, the glycosylase substrate base uracil will be generated in the amplified target nucleic acid sample. Addition of uracil DNA-glycosylase to the sample releases the uracil from the sample. In the case where the modified precursor nucleotide is dITP, the glycosylase substrate base hypoxanthine will be generated in the amplified target nucleic acid sample. Addition of alkylpurine DNA-glycosylase to the sample releases the hypoxanthine from the sample. Release of the glycosylase substrate bases from the amplified target nucleic acid sample results in an apyrimidinic site in the case of uracil and an apurinic site in the case of hypoxanthine.

Glycosylase mediated cleavage of the target strand of an amplified target nucleic acid sample as a result of a particular sequence being present or absent at the candidate locus in the amplified target nucleic acid also permits detection by immobilisation methods. In this instance one of the primers usually the adjacent primer is synthesised with a "capture" agent attached which allows immobilisation of the target strand to a solid matrix. The primers are designed so that the modified precursor nucleotide is incorporated at the candidate locus alone in the amplified target nucleic acid sample. The amplification process is carried out in the presence of a labelled precursor nucleotide resulting in the incorporation of label into the extended adjacent primer distal (3') to the candidate locus. Immobilisation of the amplified target strand is achieved by incubating the amplified target strand with a solid matrix bearing a molecule which binds the capture agent specifically. Removal of the complementary target strand is then performed by washing with a denaturing agent and removing all of the non-immobilised material. If a glycosylase substrate base is present at the candidate locus, release of the labelled portion of the target strand will occur in the glycosylase mediated cleavage process. Removal of the released fragment and determination of the level of label released or remaining immobilised is diagnostic of the presence or absence of the particular sequence at the candidate locus. Alternatively, if labelling of the target strand is not desirable, detection may be performed by use of specific nucleic acid hybridisation probes. Such probes can be readily designed to detect the complete immobilised target strand, the portion of the target strand released after the glycosylase mediated cleavage process or the portion of the target strand remaining after the cleavage process.

This glycosylase mediated cleavage process allows the detection of any particular sequence at a candidate locus in any target sample provided the DNA sequence surrounding the mutation site is known. Sequence information of at least 15 to 20 nucleotides on each side of the candidate locus is required in order to design appropriate primers for the amplification process.

The use of a DNA glycosylase enzyme which recognises a modified base has not previously been used directly for the detection of a particular sequence at a candidate locus in an amplified target nucleic acid sample.

5 A main application of the glycosylase mediated cleavage process is that it permits detection of the presence or absence of a particular sequence at a candidate locus by the aforementioned immobilisation methods.

10 A suitable "capture" agent is biotin. Thus, one of the primers is suitably synthesised with biotin attached which allows immobilisation of the target strand to streptavidin coated on, or linked to a solid matrix so as to allow immobilisation of the target strand. The primers are designed so that the modified precursor nucleotide is only incorporated in the target strand at the candidate locus alone if a particular sequence
15 is present or absent at the candidate locus.

Suitably one of the precursor nucleotide(s) is also labelled. As indicated above the amplification process is preferably carried out in the presence of a labelled precursor nucleotide, such as an alpha P³² dNTP, a fluorescent dNTP or digoxigenin dNTP resulting in the
20 incorporation of the label into the extended adjacent primer. The primer bearing the capture groups is also designed so that the labelled precursor nucleotide(s) can only be incorporated distal to (3') the primer in question and the candidate locus i.e. the labelled precursor nucleotide cannot be incorporated between the candidate locus and the
25 adjacent primer.

Preferably the phosphate linkages at the abasic sites are cleaved by a treatment selected from alkali treatment or other chemical treatment, heat treatment and treatment with an enzyme.

30 The process of strand cleavage resulting from the release of glycosylase substrate base by DNA glycosylase action followed by abasic site cleavage is referred to herein as glycosylase mediated

cleavage. The presence or absence of a particular sequence at the candidate locus results in the incorporation, or lack of incorporation of a modified precursor nucleotide at the candidate locus in the amplified target nucleic acid sample. Thus if a modified precursor nucleotide is incorporated at the candidate locus, cleavage of the target strand will occur at the candidate locus through the glycosylase mediated cleavage process and this cleavage point will be the closest cleavage point to the 3' end of the extended adjacent primer. If a modified precursor nucleotide is not incorporated at the candidate locus, the closest cleavage point to the 3' end of the extended adjacent primer will occur at the first point distal to the candidate locus where a modified precursor nucleotide is incorporated. Therefore the observed length of the extended adjacent primer following the glycosylase mediated cleavage process will be diagnostic of the presence or absence of the particular sequence at the candidate locus.

The preferred treatment is alkali at high temperature, or with an enzyme which cuts specifically at apurinic or apyrimidic sites, such as *E.coli* endonuclease IV. Both of these treatments cleave the apurinic or apyrimidic site to completion on the 5' side. In a case where there is a sole glycosylase substrate base at the candidate locus in the amplified target strand, glycosylase mediated cleavage cuts the target strand at a single position yielding two fragment strands. Manipulation of the design of the primer sequences used permits amplification of products bearing glycosylase substrate bases at any desired position(s) on the target and/or complementary strand in addition to the candidate locus on the target strand and facilitates subsequent analysis of the glycosylase cleaved amplified target nucleic acid sample.

The presence or absence of a particular sequence at the candidate locus determines whether or not a glycosylase substrate base is incorporated at the candidate locus in the target strand. Thus different diagnostic cleavage patterns are produced by glycosylase mediated cleavage of an amplified target strand depending on whether the particular sequence is present or absent at the candidate locus. Primers may also be designed so that glycosylase substrate bases are absent from

the target strand if a particular sequence is present at the candidate locus. In such a case, the target strand is resistant to glycosylase mediated cleavage.

5 The products/cleavage pattern resulting from the glycosylase mediated cleavage of the amplified target nucleic acid sample may be discerned by existing DNA sizing methods such as polyacrylamide gel electrophoresis, agarose gel electrophoresis or high performance liquid chromatography (HPLC).

10 Thus, the size of the extended adjacent primer when an amplification method is used in accordance with the invention can be determined after denaturation by existing DNA sizing methods such as denaturing polyacrylamide gel electrophoresis. Labelling of the adjacent primer prior to the amplification process facilitates detection of the cleaved extended adjacent primers alone while the use of a
15 labelled precursor nucleotide in the amplification process or DNA staining methods facilitated detection of all of the fragments generated as a result of glycosylase mediated cleavage of the amplified target nucleic acid sample.

The modified base used is preferably uracil or hypoxanthine.

20 Thus, the preferred modified precursor nucleotides are dUTP and dITP which when incorporated into DNA generate the glycosylase substrate bases uracil and hypoxanthine respectively. The modified precursor nucleotide dUTP is a base sugar phosphate comprising the base uracil and a sugar phosphate moiety. The modified precursor
25 nucleotide dITP is a base sugar phosphate comprising the base hypoxanthine and a sugar phosphate moiety. Uracil in DNA is recognised specifically by uracil DNA-glycosylase (UDG) and released from DNA. Uracil DNA glycosylase also recognises certain other uracil related bases when present in DNA. Hypoxanthine is recognised
30 specifically by alkylpurine DNA glycosylase (ADG) and released from DNA. This enzyme also recognises and releases N3 methyl adenine. N3 methyl guanine, O2 methyl cytosine and O2 methyl thymine when

present in DNA. Amplification of a target DNA sequence using the precursor nucleotides dATP, dCTP and dGTP and the modified precursor nucleotide dUTP results in an amplified DNA where thymine is replaced uracil. The uracil is incorporated in the newly synthesised DNA strand at positions complementary to adenine residues in the template DNA strand during the amplification process. Amplification of a target DNA sequence in the presence of the precursor nucleotides dATP, dCTP and dTTP and the modified precursor nucleotide dITP results in an amplified DNA where guanine is preferentially replaced by hypoxanthine. The hypoxanthine is preferentially incorporated opposite cytosines in the template DNA strand in the amplification process when the other precursor nucleotides are not limiting.

Any DNA or RNA from any source can serve as a target sample.

Preferably, the target nucleic acid sample is DNA. For amplification purposes, RNA is first converted into cDNA by reverse transcription. A particular sequence may be present at the candidate locus in all or a portion of the target nucleic acid sample, or may be absent from the target nucleic acid. The target nucleic acid sample may be homozygous or heterozygous for the presence or absence of a particular sequence at the candidate locus which is one of the advantages of the method according to the invention over some of the prior art methods discussed above.

The DNA which can be used in accordance with the method of the invention can be single stranded, homoduplex or heteroduplex DNA.

Some of the advantages of the present invention relative to specific prior art methods are as follows.

The invention has advantages over the restriction enzyme analysis method given that a minimum of two glycosylases are required to identify all possible sequence changes due to mutations such as point, insertion or deletion type mutations at a candidate locus and one

glycosylase can be used to detect 10 out of all 12 possible point mutations.

The method according to the invention is significantly faster than DNA sequencing in that full size DNA sequencing type gels are not
5 necessary for sizing the cleavage products and the number of cleavage products that need to be detected to determine if a specific sequence is present or absent in a target nucleic acid is small. Usually, the appearance or disappearance of a single additional DNA fragment in addition to the wild type fragment will be sufficient to diagnose the
10 presence or absence of the particular DNA sequence in the target nucleic acid. By contrast in DNA sequencing, resolution of multiple DNA fragments of different size is necessary to detect the presence or absence of a specific sequence at a candidate locus. The method according to the invention allows the detection of the presence or
15 absence of a specific sequence at a candidate locus by determining whether a DNA fragment is released or not from an immobilised target nucleic acid. This is not possible with DNA sequencing.

The method according to the invention also represents a significant improvement over the uracil interference method. The
20 method according to the invention differs from the uracil interference method in that the modified nucleotide introduced into the target nucleic acid sample is introduced at all preselected positions whereas in the case of the uracil interference method uracil is incorporated randomly and at a low level in the amplified molecules. Also in the
25 case of the method according to the invention the modified nucleotide introduced replaces a particular DNA precursor, while in the uracil interference method a ratio of modified nucleotide to normal nucleotide is used. Other advantages of the method according to the invention relative to the uracil interference method are that the method
30 according to the invention is significantly faster, full size DNA sequencing gels are not necessary for sizing the cleavage products, and the number of cleavage products that need to be detected is small. Usually, the appearance or disappearance of a single additional DNA fragment in addition to the wild type fragment will be sufficient to

diagnose the presence or absence of the particular DNA sequence in the target nucleic acid. By contrast in the uracil interference method, resolution of multiple DNA fragments of different size would be necessary to detect an uracil residue at a specific candidate locus. The method according to the invention allows the detection of the presence or absence of a specific sequence at a candidate locus by determining whether a DNA fragment is released or not from an immobilised target nucleic acid. This is not possible with the uracil interference method.

Advantages of the method according to the invention relative to use of mismatched nucleotide glycosylases are that external probes are not required to detect the presence or absence of a particular sequence (a mutation or otherwise) in a homozygous or heterozygous state. In the method according to the invention certain glycosylases such as uracil DNA-glycosylase work on single stranded DNA so that single stranded DNA can be investigated. Furthermore, the modified bases used in accordance with the invention are recognised and removed efficiently by the glycosylase enzymes which recognise them. Thus the invention offers significant advantages over the mismatch cleavage method outlined about in that the provision of external probes is not necessary, no hybridisation steps are required, single stranded DNA can be used and high throughput of samples can be achieved rapidly and easily.

Finally, the method according to the invention has significant advantages over the ARMS method in that only one amplification reaction is necessary to determine whether wild type or mutant sequence is present at the candidate locus. The annealing conditions for the ARMS method have to be precise, thus the method is difficult to transfer in many cases and has to be optimised for each mutation investigated. By contrast the method according to the invention is robust, and is optimised easily and enables one to achieve a high throughput of samples.

Brief Description of Drawings

Fig. 1 is a schematic representation of procedure A) described in Example 1;

5 Fig. 2 is a schematic representation of procedure B) described in Example 1;

Fig. 3 is a schematic representation of the procedure described in Example 2;

Fig. 4 is a schematic representation of the procedure described in Example 3;

10 Fig. 5 is a schematic representation of the procedure described in Example 4;

Fig. 6 is a schematic representation of the procedure described in Example 5;

15 Fig. 7 is a schematic representation of the procedure described in Example 6;

Fig. 8 is a schematic representation of the procedure described in Example 7; and

Fig. 9 is a schematic representation of the procedure described in Example 8.

20 Modes for Carrying Out the Invention

The invention will be further illustrated by the following Examples.

Example 1

The method according to the invention was used to detect the presence of a G to A base substitution mutation causal of malignant hyperthermia at position 1021 of the human skeletal ryanodine receptor gene (RYR1) in a patient heterozygous for the mutation. The sequence of steps is depicted in Fig. 1. In this case, the target nucleic acid was DNA extracted from a patient with malignant hyperthermia, the candidate locus was nucleotide 1021 in the human RYR1 gene and the objective was to determine whether the particular sequence base pair G:C or A:T was present at the candidate locus in either RYR1 allele in the patient. The lower strand shown in Fig. 1 is the target strand and the presence or absence of a T (U) nucleotide at the candidate locus was determined. The upper strand is the complementary target strand.

In the context of the Example the normal allele refers to the RYR1 allele bearing the normal sequence and the mutant allele refers to the RYR1 allele bearing the mutant sequence at position 1021. The DNA sequence surrounding the mutation site is shown in Fig. 1, with the mutation site indicated by a bold upper case letter.

A) The sequence of the target nucleic acid at the candidate locus region and the sequence of the adjacent and distal primers (primers contain standard nucleotides (dG, dA, dT and dC)) are shown in Fig. 1. Six pmoles of the adjacent primer was end-labelled by incubation with 1 unit of polynucleotide kinase, appropriate buffer and 1 μ Ci 32 P ATP (3000Ci/mmol) for 30 min at 37°C. The target nucleic acid sample was amplified by PCR as follows: the reaction mix for PCR contained 200 ng genomic DNA from the affected patient, 0.2 mM dATP, dCTP, dGTP and dUTP, 1.5mM MgCl₂, 50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% TritonX-100, 6 pmoles of each primer in a total volume of 19 μ l. The reaction mix was then overlaid with an equal volume of mineral oil and a hot start PCR was performed wherein the reaction mix was heated to 94°C for 5 min prior to addition of 1 unit of Taq polymerase (bringing the total volume to 20 μ l). 30 Cycles of 94°C for 60 sec., 59°C for 60 sec. and 72°C for

60 sec. were carried out in thermocycler followed by removal of the aqueous reaction mixture to a separate microtube. The reaction mixture bearing the amplified target nucleic acid was then treated with exonuclease I to digest the primers not extended in the amplification
5 step. This was achieved by incubating 2 μ l of the PCR reaction with 0.5 units of exonuclease I at 37°C for 30 min. The exonuclease was subsequently heat inactivated by incubating the reaction at 80°C for 15 min.

Uracil DNA glycosylase (0.05 units) was then added and the
10 incubation continued at room temperature for 30 min. Following Uracil DNA glycosylase treatment, the apurinic and apyrimidic (AP) sites generated in the amplified product were cleaved to completion by adding NaOH to a final concentration of 0.25M and heating the mixture for 15 min at 95°C. The reaction was then neutralised by addition of
15 Tris base to 30mM final concentration.

An equal volume of formamide loading dye (90% formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol) was added to the sample which was then heated at 85°C for 5 min. All of the sample was then loaded onto a 20% denaturing (7M urea) polyacrylamide gel
20 and electrophoresis was carried out for 3-4 hours at 400 volts for size analysis of the cleaved products in the sample. Following electrophoresis, autoradiography was carried out by exposing the gel directly to X-ray photographic film for 12 hours at -70°C. A labelled set of oligonucleotides (20mer, 22mer, 24mer) was used as markers.
25 Analysis of the autoradiographed products showed that two cleavage products were present in approximately equal amounts. One product was 22 nucleotides (n) in length, corresponding to the normal allele, while the second product corresponding to the mutant allele, was 20n in size, as hereinafter described.

30 The 20n product could only have been generated if an uracil residue was incorporated at position 1021 in the target strand. Thus the detection of a 20n fragment is diagnostic of the presence of a T residue at position 1021. In the normal allele the first uracil

incorporated should be at position 1019 and should result in a 22n product. Thus the detection of a 22n fragment is diagnostic of the absence of a T residue at position 1021 and the presence of a T residue at position 1019. Both the 20n and 22n product were detected in
5 roughly equal amounts indicating that the target nucleic acid was from an individual heterozygous for the G1012A mutation. If the patient was homozygous normal, then only a 22n product should be detected. If the patient was homozygous affected, then only the 20n product should be detected. The relative intensity of the 22n product to the 20n
10 product allows one to determine the relative levels of the normal and mutant allele in a target nucleic acid sample. This is especially useful for analysing complex samples where there may be a large difference between the levels of a particular normal and mutant allele.

B) Redesign of the adjacent primer so that the 3' end of the
15 primer was at position 1023, 1024 or 1025 and so that the primer maintained an appropriate optimal length (by adjusting the 5' endpoint appropriately) is permissible. For instance, if the adjacent primer is moved 3 nucleotides in the 5' direction, the primer sequence would be 5'CTG CAC GAA GCA CAG TGA CT 3'. Thus a product of 23n
20 would be generated from the mutant allele and a product of 25n would be generated from the normal allele. There would not be any need to remove the non utilised primers since the adjacent primer would be 20n in length. This modification is depicted in Fig. 2.

Example 2

25 In certain instances, it is advantageous to design the primers for amplification in accordance with the invention so that a greater and/or a clearer size difference exists between the glycosylase cleaved products diagnostic of the presence or absence of a particular sequence at a candidate locus. This can be achieved by altering the sequence of one
30 or both of the primers so that the position of incorporation of the glycosylase recognisable substrate base(s) distal to the adjacent primer and the candidate locus is altered. This can be achieved by synthesising the primers so that some or all of the residues promoting incorporation

of glycosylase substrate base(s) in the newly synthesised DNA are replaced by nucleotides which do not promote the incorporation of the glycosylase substrate base(s).

5 The protocol used in the present Example was exactly the same as that described in Example 1 except that the distal primer was synthesised with an inosine residue at the penultimate 3' position (position 1019) and exonuclease treatment was not carried out to remove the nonutilised primer as this was not necessary in this case. Thus, during amplification of the target strand a cytosine residue rather
10 than an uracil residue was incorporated opposite position 1019 in the newly synthesised DNA. As a result, glycosylase mediated cleavage of the amplified products resulted in a 23n fragment arising from the mutant allele and a 28n fragment arising from the normal allele as shown in Fig. 3.

15 Greater differences may be achieved by preventing the incorporation of additional or all uracil residues at potential positions distal to the candidate locus to the extent where cleavage of the target strand only occurs at the candidate locus if an uracil residue is present at the locus. The same approach can also be used to prevent
20 incorporation of additional or all uracil residues at potential positions opposite the adjacent primer on the complementary target strand.

Example 3

25 Cleavage of the N-glycosidic bond between a base and the DNA backbone releases the base from the DNA and results in the generation of an apurinic or apyrimidinic site (AP site). The phosphodiester bond on the 3' side of the AP site is alkali labile and is also susceptible to cleavage at neutral pH by β -elimination. Thus, treatment of a DNA sample bearing an AP site with alkali or by heating causes cleavage of the phosphodiester bond of the DNA backbone and results in a DNA
30 terminus with a 5' phosphate moiety and a terminus with a 3' deoxyribosephosphate. subsequent removal of the 3' terminal deoxyribosephosphate can be achieved by a variety of methods

including treatment with the enzyme exonuclease III or endonuclease IV. It was observed that heating of amplified DNA fragments bearing one or more AP sites for 15 min. at 95°C after uracil DNA-glycosylase cleavage resulted in cleavage of the AP site and generated products with and without a 3' terminal deoxyribosephosphate in about equal proportions. This differential cleavage may be exploited to facilitate accurate detection of the products of the glycosylase mediated cleavage process.

The protocol of Example 2 was repeated except that following uracil DNA-glycosylase treatment of the amplified target nucleic acid, the AP sites generated in the amplified product were cleaved by heating the mixture for 15 min. at 95°C and because no NaOH was added, it was not necessary to neutralise the reaction. Following completion of the protocol as described in Example 1, analysis of the autoradiograph showed four cleavage products (a 28n product and a product about 1 nucleotide larger which is the 28n product plus 3'-terminal deoxyribosephosphate (28n+) and a 23n product and a product about 1 nucleotide larger which is the 23n product plus 3'-terminal deoxyribosephosphate (23n+)) in approximately equal amounts and the nonutilised labelled primer. This modification is depicted in Fig. 4.

The 23n and 23n+ products could only have been generated if an uracil residue was incorporated at position 1021 in the target strand. Thus the detection of a 23n and 23n+ fragment is diagnostic of the presence of a T residue at position 1021. In the normal allele the first uracil incorporated should be at position 1016 and should result in a 28n product. Thus the detection of a 28n and 28n+ product was diagnostic of the absence of a T residue at position 1021 and the presence of a T residue at position 1019. In this Example, both the 23n, 23n+, 28n, and 28n+ products were detected in roughly equal amounts indicating that the target nucleic acid was from an individual heterozygous for the G1021A mutation. This approach is especially useful for a multiplex approach since conditions can be readily designed where the appearance of fragments which are larger than the nonutilised primer by a single 3'-terminal deoxyribosephosphate are

diagnostic of the presence or absence of a particular sequence at a candidate locus.

Example 4

5 Glycosylase mediated cleavage of a particular sequence at a candidate locus in a target strand of a target nucleic acid sample also permits detection of cleavage products using solid phase or immobilised technology. This feature of the invention permits high throughput of samples and avoids the use of time consuming gel electrophoresis analysis. Any candidate locus may be investigated for the presence or
10 absence of a particular sequence by this technology. The main areas for this application are in detection of gene mutations in a target sample and identification of specific organisms.

This Example and Examples 5 and 6 illustrate application in both areas as follows:

15 A procedure was used which was similar to that used in Example 1 in that the target nucleic acid was DNA extracted from a patient with malignant hyperthermia, the candidate locus was nucleotide 1021 in the human RYR1 gene and the objective was to determine if an A:T base pair was present at the candidate locus in one or both of the RYR1
20 alleles in the patient. The procedure is depicted in Fig. 5. In this case, the lower strand was the "target strand" and the upper strand was the "complementary strand". The presence or absence of a T(U) nucleotide at the candidate locus on the target strand was determined.

25 The adjacent and distal primers used were the same as those used in Example 1 except that the adjacent primer is biotinylated at the 5' end, and in the distal primer, all of the A residues were replaced by inosines. As a result of replacement of the A residues, the amplified target strand would only have contained a single uracil residue if an A:T base pair was present at the candidate locus. Thus cleavage of the
30 target strand by uracil DNA-glycosylase mediated cleavage could only occur at a single position (1021) if a uracil residue was present.

Therefore, cleavage of the amplified target strand in this instance is diagnostic of the presence of an A:T base pair at position 1021 in the target nucleic acid sample.

5 The adjacent primer was biotinylated at the 5' end during synthesis. The target nucleic acid sample was amplified by PCR as follows: the reaction mix for PCR contained 200 ng genomic DNA from the affected patient, 0.2 mM dCTP, dGTP, dATP and dUTP, 10 1 μ Ci alpha-³²P dCTP (3000Ci/mmol), 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.01% Triton X-100, 6 pmoles of each primer in a total volume of 9 μ l. The reaction mix was then overlaid with an equal volume of mineral oil and a hot start PCR was performed wherein the reaction mix was heated to 80°C for 5 min prior to addition of 1 unit of Taq polymerase (bringing the total volume to 25 15 μ l). 30 Cycles of 94°C for 60 sec., 59°C for 60 sec. and 72°C for 60 sec. were carried out in a thermocycler.

Immobilisation of the product was carried out by adding 1 μ l of the aqueous PCR reaction to a tube, labelled tube A, containing 20 μ l streptavidin coated magnetic beads (Dynal beads, Dynal is a trade 20 mark) and 19 μ l sterile water and incubating at room temperature for 10 minutes. The tube was then placed in a magnetic stand which caused the beads with the attached biotin labelled PCR product to accumulate against one side of the tube. The supernatant containing unincorporated label and unused distal primer and nucleotide triphosphates was removed and the beads with trapped PCR amplified 25 DNA were washed several times in buffer containing 10 mM Tris-HCl

pH 7.5, 1 mM EDTA and 1M NaCl. The amplified DNA was then denatured by adding 40 μ l of 0.1M NaOH. The complementary strand was removed in the supernatant and the target strand remained attached to the streptavidin coated magnetic beads. The beads were again 30 washed with 40 μ l of 0.1M NaOH followed by a wash in the aforementioned buffer. The immobilised target strand was then treated with 0.05 units uracil DNA-glycosylase and cleaved at the AP-site by adding alkali to 0.25M NaOH final concentration. The released

material was then removed to another tube, labelled tube B, and monitored for the presence of ^{32}P by scintillation counting. The tube containing the cleaved immobilised PCR product was also monitored for the presence of ^{32}P .

- 5 Monitoring for the present of ^{32}P by scintillation counting demonstrated that following uracil DNA-glycosylase cleavage, about half of the radioactive material was released to tube B. This demonstrated that about half of the immobilised PCR product was
10 cleaved due to the presence of an U residue at the candidate locus. Thus it could be concluded that the target sample was heterozygous for the A:T base pair at position 1021 in the RYR1 gene.

Example 5

- Glycosylase mediated cleavage with immobilisation was used to detect the presence of pathogenic organisms of the *Mycobacterium*
15 *tuberculosis* complex.

- The MPB70 gene is specifically found in *Mycobacterium tuberculosis* complex organisms and several reports have been published where detection of the MPB70 gene by PCR or other means can be used successfully to diagnose the presence of the complex. In
20 this example, specific amplification of a section of the MPB70 gene was carried out so that a single U and label was incorporated in the PCR product. Thus, immobilisation of the product, followed by cleavage with uracil DNA-glycosylase should have released labelled product which could readily be detected.

- 25 As shown in Fig. 6, specific primers were designed to amplify a section of the MPB70 gene so that the amplified section of the target strand delineated by the primers contained several guanine, cytosine or adenine nucleotides and a single uracil residue. To achieve this, the distal primer was designed so that no uracils were incorporated
30 opposite it on the target strand. Following amplification using an adjacent primer with a capture agent and a labelled nucleotide

precursor, the amplified target strand was immobilised and cleaved with uracil DNA-glycosylase. Thus a labelled fragment of the target strand was released by this process and could be readily detected.

5 More specifically, the objective in this Example was to detect the presence of an amplified fragment of MPB70 (nucleotide 472-540) by monitoring the cleavage of an uracil residue at position 496,
10 incorporated during extension of the adjacent primer. Primers were designed so that a GCA rich sequence (target strand) within the MPB70 gene was amplified using dGTP, dCTP, dUTP and dATP. The distal primer was designed so that inosines (or mismatches) were substituted
15 for adenine residues thereby ensuring that the only uracil present in the target strand of the amplified product was at position 496. The adjacent primer was biotinylated at the 5' end. The sequence of the target nucleic acid at the candidate locus region and the sequence of both the adjacent primer and the distal primer are shown in Fig. 6.

The target nucleic acid sample was amplified by PCR as follows: the reaction mix for PCR contained 200 ng genomic DNA (or 1 µl of a 1/200 dilution of a PCR amplified region of the MPB70 gene), 0.2 mM dATP, dCTP, dGTP and dUTP, and 1 µCi $\alpha^{32}\text{P}$ dCTP
20 (3000/Ci/mmol), 1.5 mM MgCl_2 , 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 6 pmoles of each primer in a total volume of 9 µl. The reaction mix was then overlaid with 20 µl of mineral oil and a hot start PCR was performed wherein the reaction mix was heated to 94°C for 5 min prior to addition of 1 unit of Taq polymerase (bringing
25 the total volume to 10 µl). 30 Cycles of 94°C for 60 sec., 58°C for 60 sec. and 72°C for 60 sec. were carried out in a thermocycler followed by removal of the aqueous reaction mixture to a separate microtube.

30 Immobilisation of the product was carried out by adding 1 µl of the aqueous PCR reaction to a tube, labelled tube A, containing 20 µl streptavidin coated magnetic beads (Dynal beads) and 19 µl sterile water and incubating at room temperature of 10 min. The tube was then placed in a magnetic stand which caused the beads with the attached biotin labelled PCR product to accumulate against one side of

the tube. The supernatant containing unincorporated label and unused distal primer and nucleotide triphosphates was removed and the beads with trapped PCR amplified DNA were washed several times in buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 1M NaCl. The amplified DNA was then denatured by adding 40 μ l of 0.1 M NaOH. The complementary strand was removed in the supernatant and the target strand remained attached to the streptavidin coated magnetic beads. The beads were again washed with 40 μ l of 0.1M NaOH followed by a wash in the aforementioned buffer. The immobilised target strand was then treated with 0.05 units uracil DNA-glycosylase and cleaved at the AP-site by adding alkali to 0.25 M NaOH final concentration. The released material was then removed to another tube, labelled tube B, and monitored for the presence of ^{32}P by scintillation counting. The tube containing the cleaved immobilised PCR product is also monitored for the presence of ^{32}P .

Monitoring for the presence of ^{32}P by scintillation counting demonstrated that following uracil DNA-glycosylase cleavage, the radioactive material was released to tube B. This demonstrated that the immobilised PCR product was cleaved due to the presence of an U residue at the candidate locus. Thus it could be concluded that the target sample originated from *Mycobacterium tuberculosis* complex.

Example 6

The procedure of Example 5 was repeated except that the reaction was carried out using the adjacent primer designed in a different way. In this case, the adjacent primer was designed so that no uracils were incorporated into the target stand during amplification (shown in Fig. 7). Thus, in this case, the amplified product should have been resistant to cleavage with uracil DNA-glycosylase and so the label should not have been released to tube B. In this case, monitoring for the presence of ^{32}P by scintillation counting demonstrated that the label in tube B remained immobilised after incubation with uracil DNA-glycosylase. No label was released to tube B. This application is especially useful for verifying that the correct DNA is immobilised

since spurious PCR products generated would be expected to have uracil residues incorporated and thus, label should be released to tube B. The combination of Examples 5 and 6 allows the investigator to diagnose the presence or absence of *Mycobacterium tuberculosis* complex with a high degree of confidence.

Example 7

Amplification of specific fragments of DNA of a specific size using preselected primers is commonly used for diagnostic purposes in the DNA diagnostics area. One of the limitations of such a diagnostic approach is that artefactual amplification products of the same size as the diagnostic product in question can arise and can potentially result in misdiagnosis. Thus, a method which increases the confidence limit or verifies that a diagnostic amplified fragment is correct has significant value in the diagnostic area since it improves the accuracy of diagnosis and is superior to diagnosis by amplification alone.

Amplification of specific fragments of the MPB70 gene found in *Mycobacterium tuberculosis* complex organisms using specific primers have been used to diagnose the presence of these organisms in samples. In this Example, the method according to the invention was used to check that a 116bp and a 130bp fragment of the MPB70 gene amplified from *Mycobacterium tuberculosis* complex using specific primers was correct. First of all, PCR was employed using specific primers and a *Mycobacterium tuberculosis* complex DNA sample so that a 116bp and 130 bp product was amplified from the MPB70 gene. The primers and amplification conditions were designed so that when the 116bp and 130bp amplification products were cleaved with uracil DNA-glycosylase, fragments of a specific size should be generated verifying that the initial amplification products were correct.

The amplification strategy is shown in Fig. 8. As can be seen, primer A (5'ggcctcggtgcaggaatgtc3') and B (5'ccaggtttacttgcggattga3') were selected to amplify a 116bp region of the MPB70 gene encompassing nucleotide 320 to nucleotide 436. The primers were also

selected so that the first uracil incorporated was 11 nucleotides downstream of the 3' end of the primer A and 2 nucleotides downstream of primer B. Similarly, primer C and D were selected to amplify a 130bp region of the MPB70 gene encompassing nucleotide
5 551 to nucleotide 681. The first uracil incorporated in this case was 26 nucleotides downstream of the 3' end of the primer C and 2 nucleotides downstream of primer D. Amplification of the 116bp and 130bp of the MPB70 gene using labelled primers, followed by cleavage with uracil-DNA-glycosylase should result in a 32n and a 23n fragment from the
10 116bp PCR product and a 47n and a 23n fragment from the 130bp PCR product.

The 116bp product was amplified from a *Mycobacterium tuberculosis* complex DNA sample by PCR as follows. The reaction mix for PCR contained 200 ng *Mycobacterium tuberculosis* complex
15 DNA, 0.2mM dATP, dCTP, dGTP and dUTP, 1.5mM MgCl₂, 50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100 and 6 pmoles of primer A and primer B in a total volume of 19µl. In the first amplification reaction mix, primer A was end-labelled using γ^{32} ATP and polynucleotide kinase, while primer B was labelled in the second
20 amplification. The reaction mixes were then overlaid with an equal volume of mineral oil and a hot start PCR was performed wherein the reaction mixes were heated to 94°C for 5 min prior to addition of 1 unit of Taq polymerase (bringing the total volume to 20µl). Thirty cycles of 94°C for 60 sec., 57°C for 60 sec. and 72°C for 60 sec.
25 were carried out in a thermocycler followed by removal of the aqueous reaction mixes to separate microtubes. The reaction mixes bearing the amplified target nucleic acids were then treated with exonuclease I to digest the primers not extended in the amplification step. This was achieved by incubating 3µl of the PCR reaction mixes with 0.5 units of
30 exonuclease I at 37°C for 30 min. The exonuclease was subsequently heat inactivated by incubating the reaction at 80°C for 15 min.

Uracil DNA glycosylase (0.05 units) was then added to 3µl of the above reaction mixes and incubated at 37°C for 30 min. Following Uracil DNA glycosylase treatment, the apyrimidinic sites generated in

the amplified products were cleaved to completion by adding NaOH to a final concentration of 0.05M and heating the mixtures for 15 min at 95°C. The reaction mixes were then neutralised by addition of Tris base to 0.03M final concentration. An equal volume of formamide loading dye (90% formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol) was added to the mixes which were then heated to 85°C for 5 min. 5µl of each mix was then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried out for 3 hours at 400 volts for size analysis of the cleaved products in the sample. Following electrophoresis, autoradiography was carried out by exposing the gel directly to X-ray photographic film for 12 hours at -70°C.

Analysis of the autoradiographed products showed the following cleavage pattern. In the first reaction where primer A was end-labelled, a product of 32n was observed. The 32n product could only have been generated if an uracil residue was incorporated 12 nucleotides downstream of the 3' end of the primer A. In the second reaction where primer B was end-labelled, a product of 23n was observed. The 23n product could only have been generated if an uracil residue was incorporated 3 nucleotides downstream of the 3' end of primer B.

The 130bp product was amplified from a *Mycobacterium tuberculosis* complex DNA sample by PCR exactly as outlined above except that primer C (5'catcctgacctaccacgtagt3') and primer D (5'gtcggcggtaccgaccttgag3') were used in place of primer A and primer B. Analysis of the autoradiographed products showed the following cleavage pattern. In the reaction where primer C was end-labelled, a product of 47n was observed. The 47n product could only have been generated if an uracil residue was incorporated 27 nucleotides downstream of the 3' end of primer A. In the reaction where primer D was end-labelled, a product of 23n was observed. The 23n product could only have been generated if an uracil residue was incorporated 3 nucleotides downstream of the 3' end of primer D.

The amplification process described here can be performed independently with separately labelled primers as described or as a multiplex reaction where all four labelled primers are included in a single amplification reaction. In the latter case, labelled products of
5 47n, 32n and 23n are detectable in a single lane following cleavage with uracil-DNA-glycosylase, electrophoresis and autoradiography.

The probability of an uracil being present at a particular location in a DNA fragment is $1/4$ or 0.25 . The probability of an uracil not being present at a location is $3/4$ or 0.75 . In the case of primer A, it
10 was extended 11 nucleotides before it was cut. The probability of such an extension occurring randomly is $0.75^{11} = 0.042$. The extended primer cut at the 12 nucleotide incorporated showing that an uracil was present at this location. The probability of the uracil occurring at that particular position was 0.25 . Thus overall, the probability of an eleven
15 base pair extension product occurring randomly would be $0.042 \times 0.25 = 0.0105$. Similarly, the probability of primer B extending by two bases would be $0.75^2 \times 0.25 = 0.14$. Overall, the probability of a random 116bp product cleaving to a 32n (11 nucleotide extension of the 21n primer A) and a 23n (2 nucleotide extension of the 21n primer
20 B) product would be $0.0105 \times 0.14 = 0.0014$. Similar calculations performed for the 130bp product where primer C and D are extended by 26n and 2n respectively show that the probability of such extension products arising randomly is 0.000019 . Taking both PCR products together, the probability of the observed products occurring randomly
25 is 0.000000027 . Thus, this Example demonstrates the application of the method according to the invention in the verification of PCR products from *Mycobacterium tuberculosis*. Thus the application of the method according to the invention in this manner offers a rapid and accurate means for verification of amplified nucleic acids from
30 diagnostic or other purposes.

Example 8

The fact that Uracil-DNA-glycosylase is active on both single stranded and double stranded DNA allows the use of this enzyme in the analysis of linear amplification products bearing uracils residues.

5 Linear amplification of single stranded DNA can offer significant advantages over exponential DNA amplification in several instances. However, linear amplification suffers from some drawbacks since single stranded DNA generated by linear amplification is generally less amenable to rapid analysis by comparison with double
10 stranded DNA. For example, single stranded DNA is generally not cleaved by restriction enzymes. Furthermore, exponential amplification of genomic DNA using a specific pair of primers in PCR results in the production of a double stranded DNA product of a specific size facilitating analysis by sizing technology. The 5' and 3'
15 ends of the fragment are defined by the forward and reverse primer. By contrast linear amplification of genomic DNA produces fragments with defined 5' ends but with undefined 3' ends. In this Example, we demonstrate the use of the method according to the invention to generate single stranded fragments of a specific size following linear
20 amplification. Amplification of a nucleic acid fragment bearing a candidate locus is usually required to determine if a particular sequence is present or absent at the candidate locus. Often it is necessary or desirable to examine multiple candidate loci in amplified DNA for the presence of particular sequences. This is especially desirable for
25 detection of mutations in genes causing human disease. Thus, any method allowing simultaneous amplification of multiple candidate loci followed by simultaneous analysis of the candidate loci is advantageous by comparison with single locus analysis. Amplification of a nucleic acid fragment(s) bearing multiple candidate loci can be achieved by
30 linear amplification or exponential amplification methods. However, the analysis of the multiple loci is often rate limiting requiring several independent approaches or full scale DNA sequencing. In this Example we also demonstrate the application of the method to simultaneously

determine if particular mutations are present or absent at two specific candidate loci.

The method according to the invention was used to simultaneously detect the presence or absence of a G to A base substitution mutation causal of malignant hyperthermia at the candidate loci 7301 and 7370 in the human skeletal ryanodine receptor gene (RYR1) in two patients heterozygous for either mutation. The sequence of steps is illustrated in Fig. 9. In this case, the target nucleic acid was a DNA fragment amplified by PCR from skeletal muscle cDNA from two patients (A and B) with malignant hyperthermia. The lower strand shown in Fig. 9 is the target strand. Primer A (26n, 5'ggcttgattagatgcatctctggtg3') was designed so that the first uracil incorporated into the extended primer in a linear amplification of the target DNA would be positioned at the sixth nucleotide incorporated in the case of the mutant allele and at the eighth nucleotide in the case of the normal allele. Primer B (28n, 5'gaattccaaggtcctccaagggcacaag3') was designed so that the first uracil incorporated would be positioned at the fifth nucleotide incorporated in the case of the mutant allele and at the twelfth nucleotide in the case of the normal allele. Both primers were extended in the same direction by linear amplification. The presence or absence of a T(U) at the candidate loci was determined by cleavage with uracil-DNA-glycosylase, gel electrophoresis and autoradiography. The target nucleic acid can be genomic DNA or any amplified section of DNA. In this Example, the target nucleic acid was generated by amplifying the 6995 to 7402 region of the RYR1 gene from cDNA using PCR.

Primers A and B were end-labelled with $\gamma^{32}\text{P}$ as described in Example 1. The target nucleic acid from patient A and patient B was treated with an equal volume of exonuclease I (0.5 units/ μl) at 37°C for 30 min to degrade primers not utilised in the PCR. The exonuclease was subsequently inactivated by incubation at 80°C for 15 min. This treatment was necessary to prevent any exponential amplification in subsequent steps. This step is not necessary when genomic DNA is the target nucleic acid. 1 μl of the target nucleic acid samples were

incubated independently with 6 pmol of labelled primer A and labelled primer B, 0.2 mM dATP, dCTP, dGTP and dUTP, 2mM MgSO₄, 10mM KCl, 20mM Tris-HCl pH 8.8, 10mM(NH₄)₂ SO₄, 0.1% Triton X-100 in a total volume of 19µl. The reaction mixes were then
5 overlaid with an equal volume of mineral oil and a hot start to the linear amplification process was performed wherein the reaction mix was heated to 94°C for 5 min prior to addition of 0.5 units of Vent DNA Polymerase (exo) (bringing the total volume to 20µl). Thirty cycles of 94°C for 60 sec., 55°C for 60 sec. and 72°C for 60 sec. were
10 carried out in a thermocycler followed by removal of the aqueous reaction mixture to a separate microtube.

Uracil-DNA-glycosylase (0.05 units) was then added to 5µl of the above reactions and incubated at 37°C for 30 min. Following uracil-DNA-glycosylase treatment, the apyrimidinic sites generated in
15 the amplified product were cleaved to completion by adding NaOH to a final concentration of 0.05M and heating the mixtures for 15 min at 95°C. The reactions were then neutralised by addition of Tris base to 0.03M final concentration. An equal volume of formamide loading dye (90% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol)
20 was added to the samples which were then heated to 85°C for 5 min. 5µl of the samples were then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried out for 3 hours at 400 volts for size analysis of the cleaved products in the sample. Following electrophoresis, autoradiography was carried out by
25 exposing the gel directly to X-ray photographic film for 12 hours at -70°C.

Analysis of the sample from patient A showed cleavage products of 39n, 33n and 31n in addition to the 26n and 28n non utilised primers. This pattern of cleavage products could only be produced if
30 patient A was homozygous for the normal allele of the RYR1 gene at candidate locus 7370 and heterozygous for the G7301A mutation. Analysis of the sample from patient B showed cleavage products of 39n, 33n and 32n in addition to the 26n and 28n non utilised primers.

This pattern of cleavage products could only be produced if patient B was homozygous for the normal allele of the RYR1 gene at candidate locus 7301 and heterozygous for the G7370A mutation. Thus, the application of the method according to the invention has permitted
5 simultaneous analysis of two candidate loci for the presence or absence of specific mutations. In this Example, two candidate loci have been investigated. The method can readily be applied to multiple candidate loci in genomic or amplified target DNA as long as the primers chosen support linear amplification but do not permit exponential
10 amplification.

Generation of amplified nucleic acid fragments of specific size is regularly used in diagnosis of infectious agents and in the verification of amplified nucleic acid molecules. In this Example, the use of the method according to the invention generated single stranded fragments
15 of a specific size following linear amplification. This is a useful application of the method since it permits the use of linear amplification rather than exponential amplification to generate amplified fragments of a specific size.

Claims:-

1. A method for rapidly detecting the presence or absence of a particular nucleic acid sequence at a candidate locus in a target nucleic acid sample which comprises the steps of:
 - 5 i) introducing a modified base which is a substrate for a DNA glycosylase into said candidate locus at one or more preselected positions;
 - ii) excising the modified base by means of said DNA glycosylase so as to generate an abasic site;
 - 10 iii) cleaving phosphate linkages at abasic sites generated in step ii); and
 - iv) analysing the cleavage products of step iii) so as to identify in said target nucleic acid sequence the presence or absence of said particular nucleic acid sequence at said candidate
15 locus.
2. A method according to Claim 1, wherein the candidate locus is amplified using a combination of normal DNA precursor nucleotides and at least one modified precursor nucleotide.
3. A method according to Claim 2, wherein at least one of the
20 primers for the amplification is positioned adjacent to the candidate locus.
4. A method according to Claim 3, wherein the at least one primer is labelled.
5. A method according to any one of Claims 2-4, wherein at
25 least one of the precursor nucleotide(s) is labelled.

6. A method according to any preceding claim, wherein the modified base is introduced by chemical modification of an existing base at the candidate locus.
- 5 7. A method according to any one of Claims 1-6, wherein the modified base is excised by means of a DNA glycosylase enzyme.
8. A method according to Claim 7, wherein the DNA-glycosylase enzyme is uracil DNA-glycosylase.
9. A method according to Claim 7 or 8, wherein the substrate for the DNA glycosylase enzyme is immobilised.
- 10 10. A method according to any preceding claim, wherein the phosphate linkages at the abasic sites are cleaved by treatment with alkali.
11. A method according to any one of Claims 1-9, wherein the phosphate linkages at the abasic sites are cleaved by a heat treatment.
- 15 12. A method according to any one of Claims 1-9, wherein the phosphate linkages at the abasic sites are cleaved by treatment with an enzyme.
13. A method according to Claim 12, wherein the enzyme is an enzyme which cleaves specifically at apurinic or apyrimidinic sites.
- 20 14. A method according to any preceding claim, wherein the modified base is uracil or hypoxanthine.
15. A method according to any preceding claim, wherein the target nucleic acid sample is DNA.
- 25 16. A method according to Claim 15, wherein the DNA is single stranded, homoduplex or heteroduplex DNA.

17. A method according to any one of Claims 1-14, wherein the target nucleic acid sample is RNA.

18. A method according to Claim 17, when dependent on Claim 2, wherein the RNA is converted into a cDNA by reverse
5 transcription prior to the amplification step.

19. A method according to any preceding claim, wherein the cleavage products are detected by hybridisation with specific nucleic acid probes.

20. A test kit or pack for carrying out a method according to
10 any one of Claims 1-19.

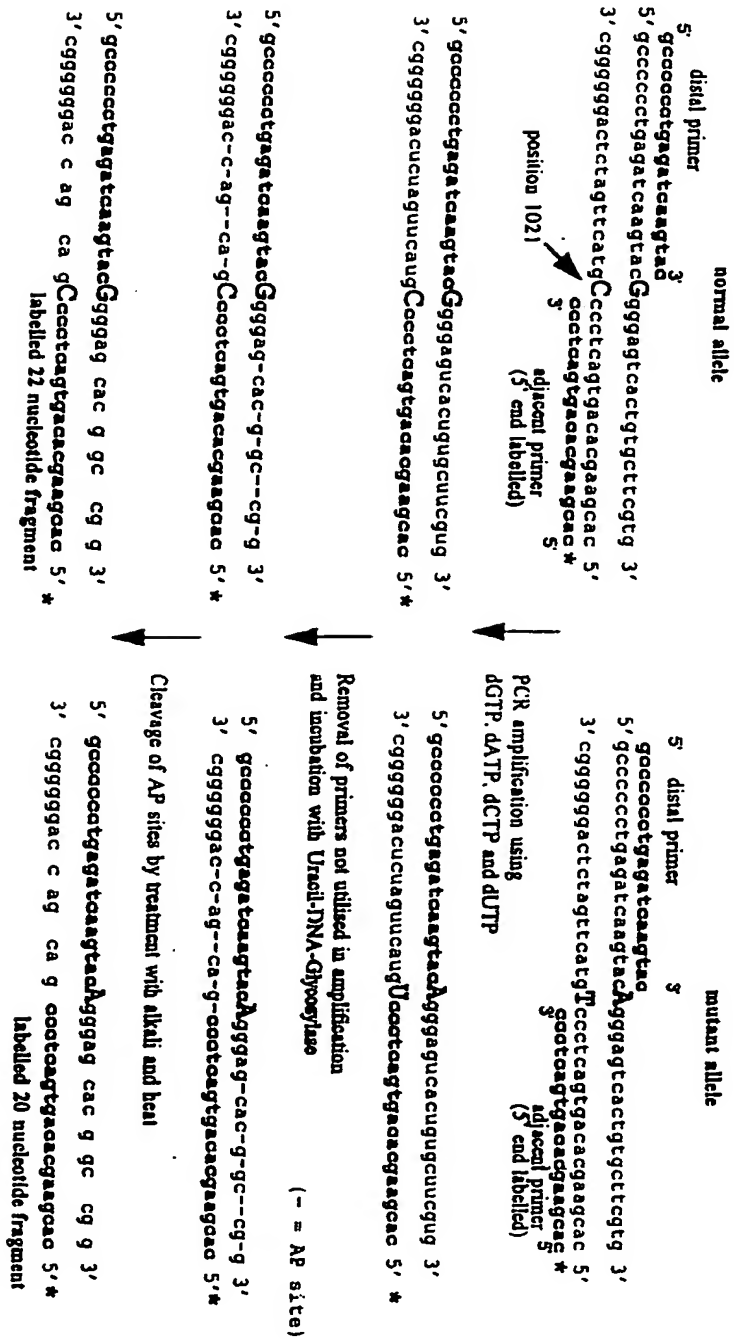
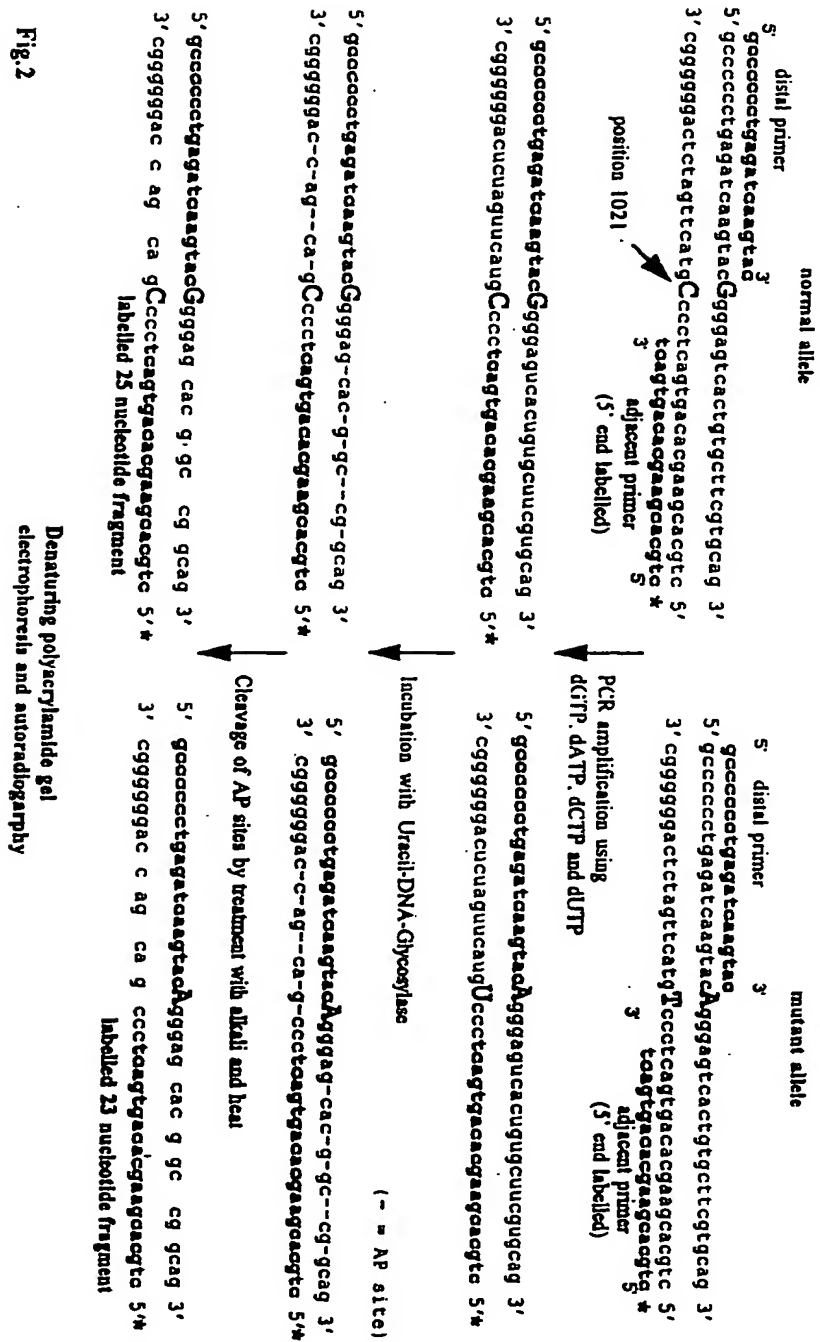


Fig.1
Denaturing polyacrylamide gel electrophoresis and autoradiography



3/9

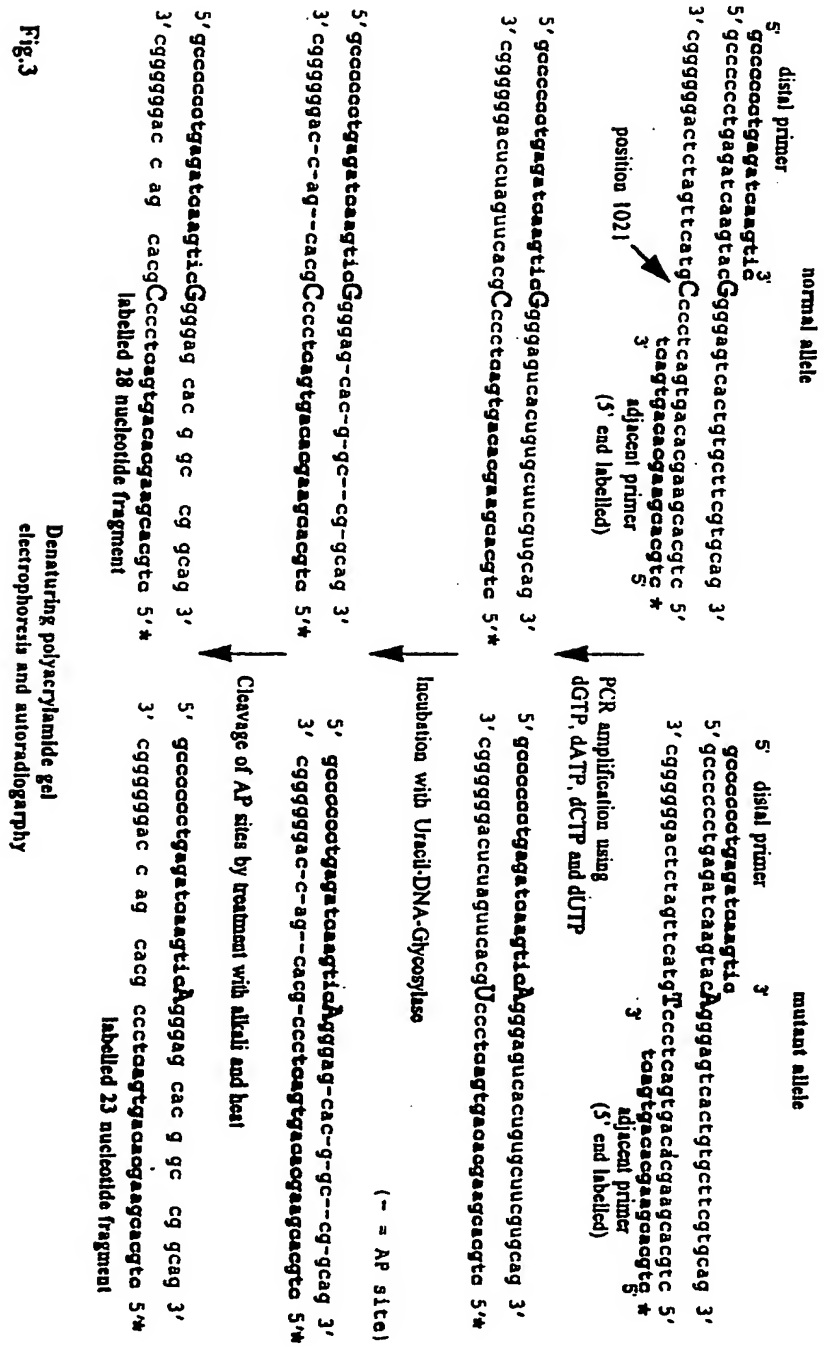
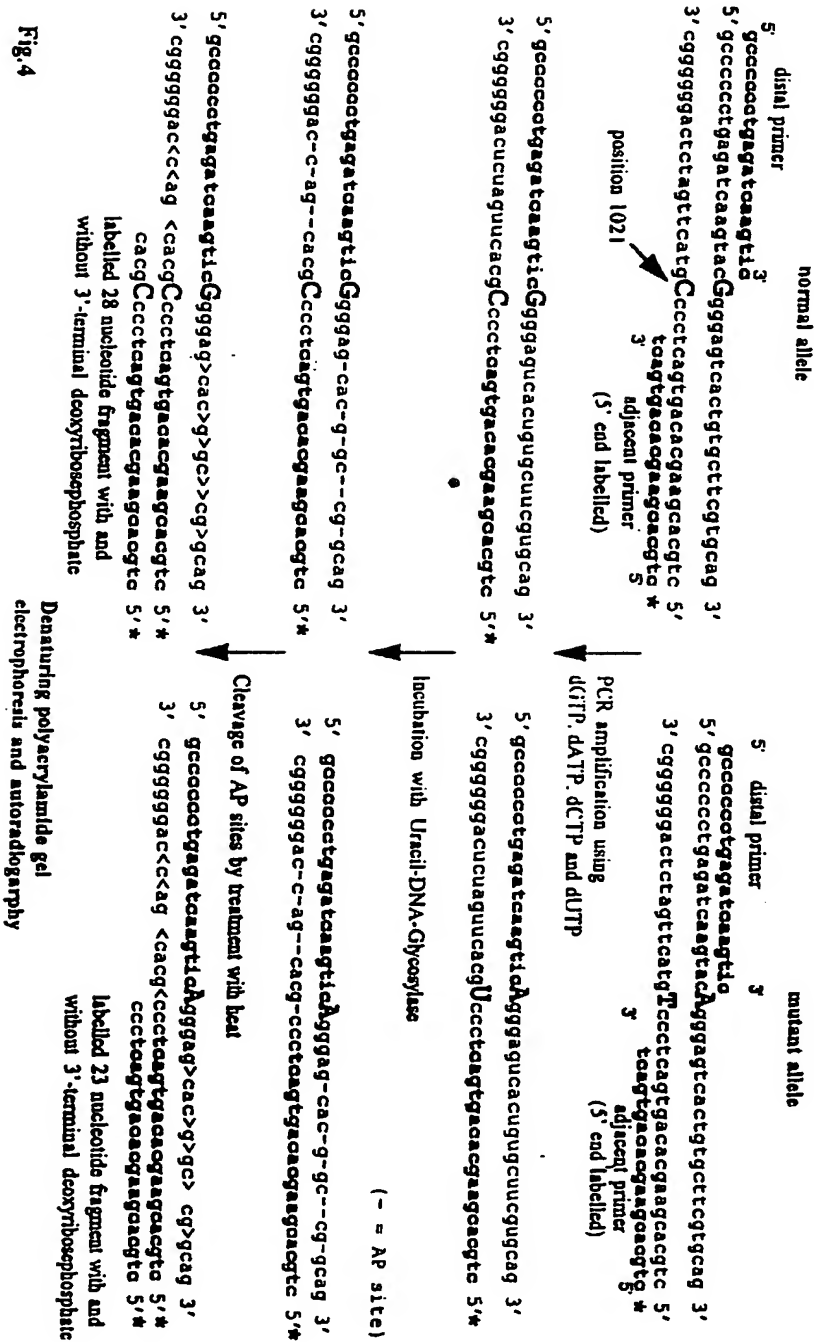
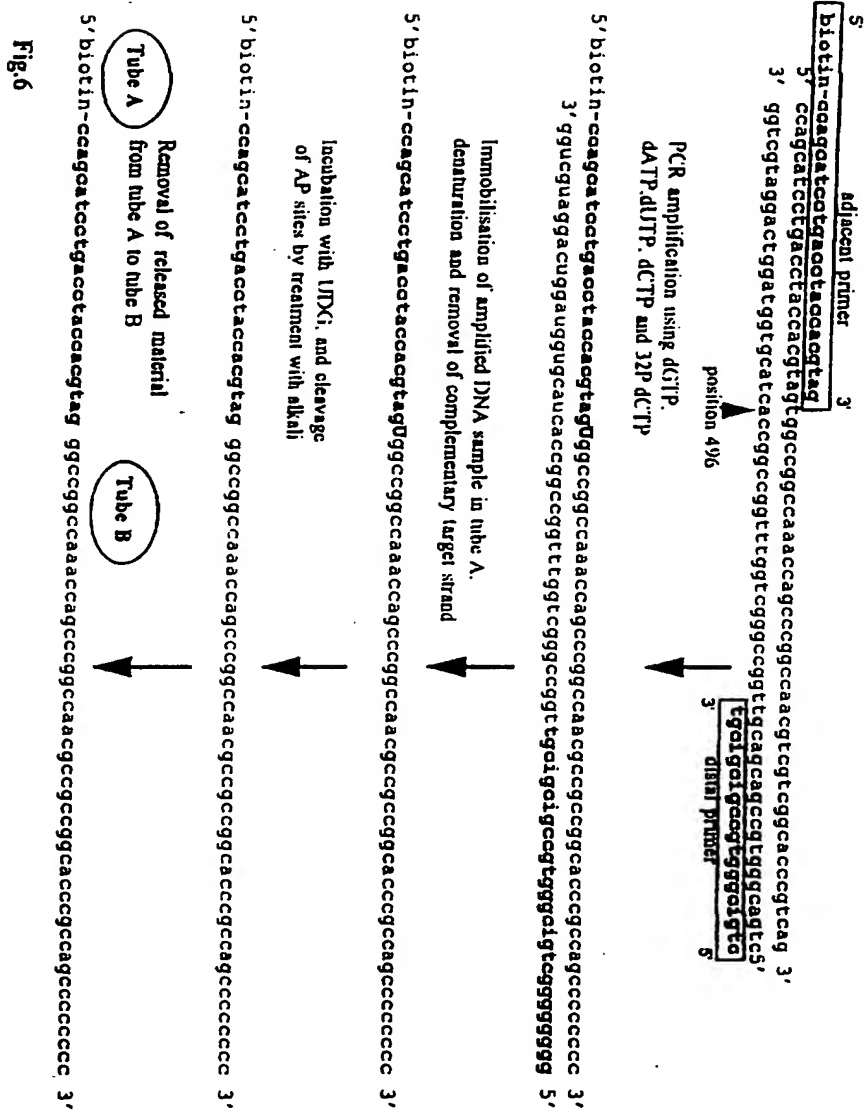


Fig.3





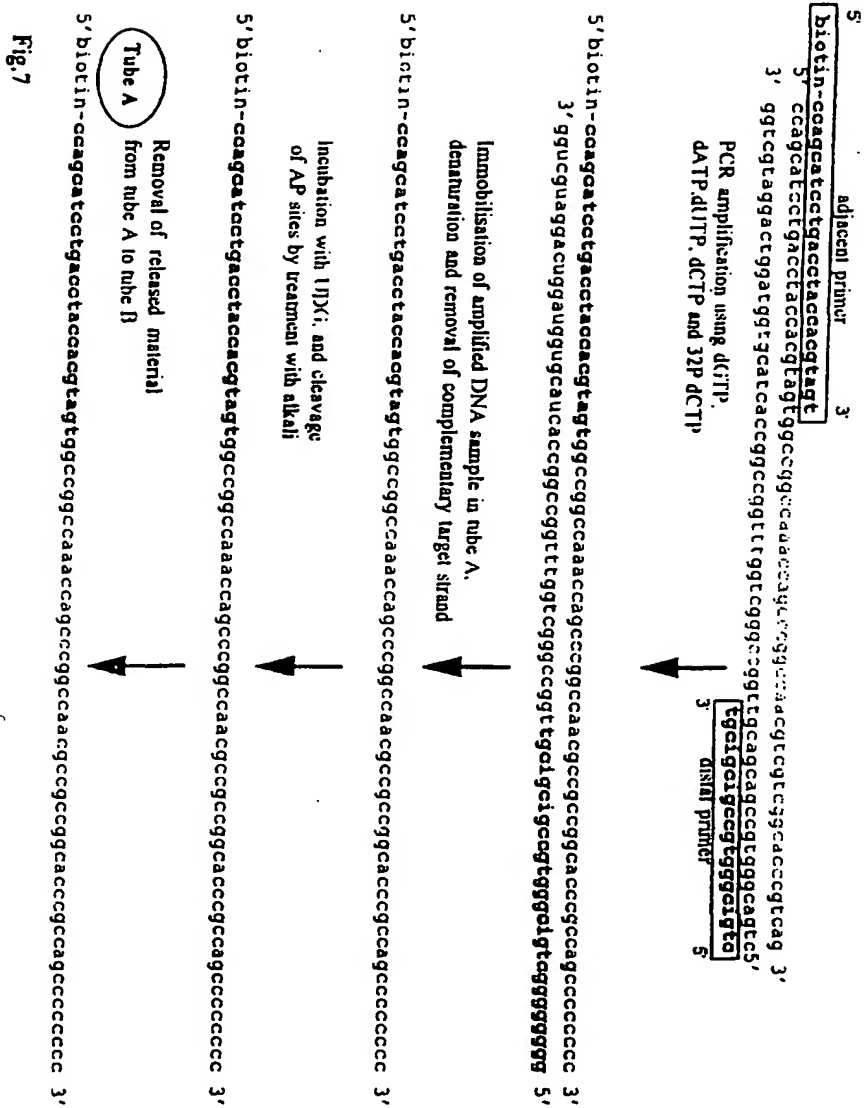
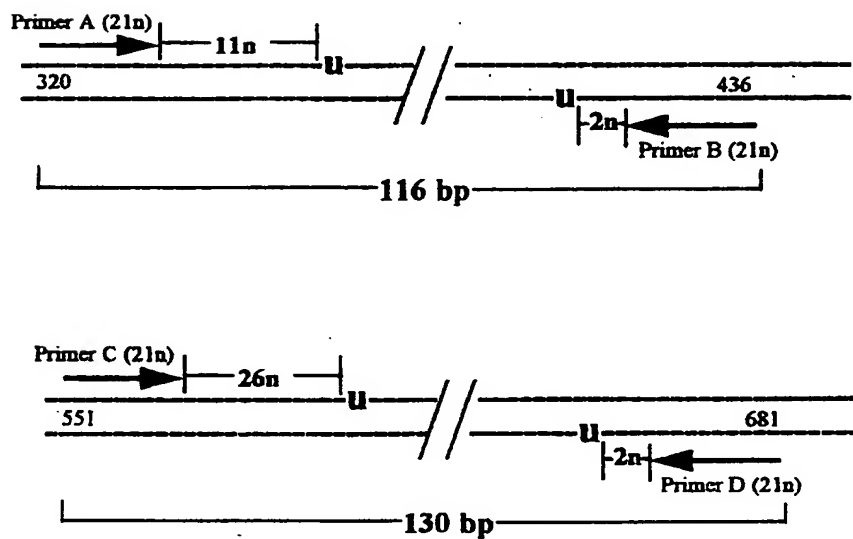


Fig. 7

8/9

M. tuberculosis MPB70 gene**Fig.8**

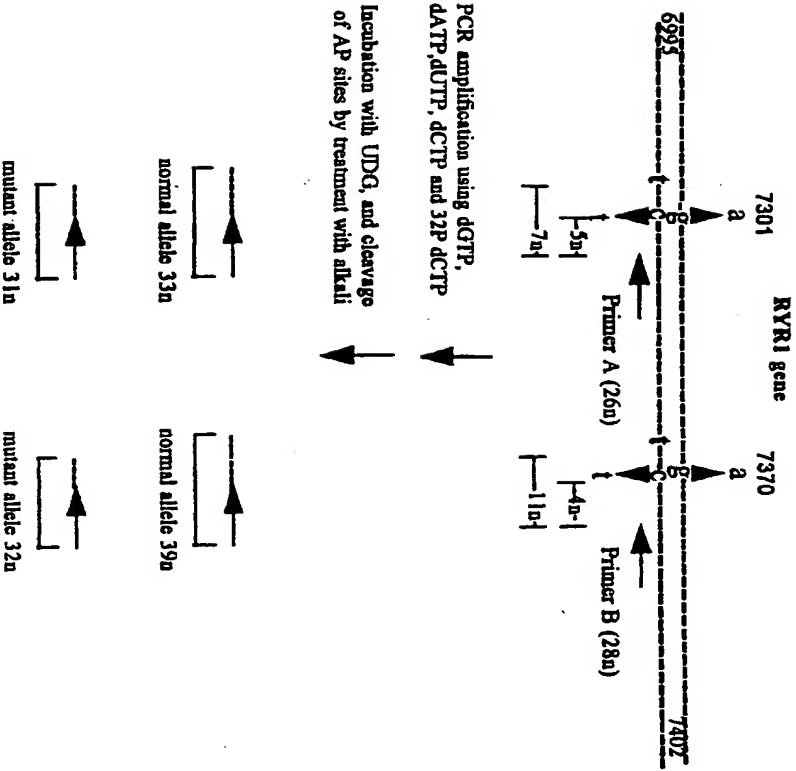


Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No
PC, IE 95/00067

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	METHODS IN MOLECULAR BIOLOGY, vol. 9, 1991, HUMANA PRESS INC., CLIFTON, NJ, US, pages 51-68, XP002004234 D.R. BENTLEY ET AL.: "Rapid methods for detection of polymorphic markers in genomic DNA" see the whole document --- -/--	1,2,4,7, 8,13-15

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

30 May 1996

Date of mailing of the international search report

07.06.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2220 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Hornig, H

INTERNATIONAL SEARCH REPORT

International Application No

PC1/IE 95/00067

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, vol. 20, no. 4, 25 February 1992, IRL PRESS LIMITED, OXFORD, ENGLAND, pages 771-775, XP002004235 W.T. PU AND K. STRUHL: "Uracil interference, a rapid and general method for defining protein-DNA interactions involving the 5-methyl group of thymines: The GCN4-DNA complex" cited in the application see the whole document ---	1,2,4,7, 8,13-15
Y	NUCLEIC ACIDS RESEARCH, vol. 21, no. 15, 25 July 1993, IRL PRESS LIMITED, OXFORD, ENGLAND, pages 3437-3443, XP002004236 P.R. DEVCHAND ET AL.: "Uracil-DNA glycosylase as a probe for DNA interactions" cited in the application see the whole document ---	1,2,4,7, 8,13-15
A	WO,A,92 01814 (CETUS CORP) 6 February 1992 see the whole document ---	1-20
A	ANALYTICAL BIOCHEMISTRY, vol. 206, no. 1, 1 October 1992, pages 91-97, XP000311343 AYOUB RASHTCHIAN ET AL: "URACIL DNA GLYCOSYLASE-MEDIATED CLONING OF POLYMERASE CHAIN REACTION-AMPLIFIED DNA: APPLICATION TO GENOMIC AND CDNA CLONING" see the whole document ---	1-20
A	PROGRESS IN NUCLEIC ACID RESEARCH AND MOLECULAR BIOLOGY, vol. 22, 1963, NEW YORK, US, pages 135-192, XP002004237 T. LINDAHL: "DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair" see the whole document ---	1-20
A	GENOMICS, vol. 14, no. 2, October 1992, ACADEMIC PRESS INC., NY, US, pages 249-255, XP000571637 A.-L. LU AND I.-C. HSU: "Detection of single DNA base mutations with mismatch repair enzymes" cited in the application see the whole document -----	1-20

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IE 95/00067

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WD-A-9201814	06-02-92	AU-B- 665338	04-01-96
		AU-B- 8532791	18-02-92
		CA-A- 2087724	25-01-92
		EP-A- 0540693	12-05-93
		US-A- 5310652	10-05-94
		US-A- 5418149	23-05-95
		US-A- 5466591	14-11-95
		JP-T- 6501612	24-02-94
